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Effect of fermented earthworm cast on egg production and egg quality as well as removal of odor in feces from egg laying hens

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Abstract

The objective of the present study was to determine the effect of feeding fermented earthworm casts (EEC) to layers on egg-laying performance, blood lipid profiles, cecal microflora, and fecal odor removing performance. A total of 200 Hyline Brown layer chicks at 33-week-old were used in this study. They were randomly assigned to two numerically equal groups with 100 replications per treatment for 10 weeks. All the birds were caged individually. The control group was not treated with EEC. The EEC group was treated with top dressing containing 3.5% EEC. The present study revealed that egg production and egg weight were increased after feeding diet containing EEC at the top dressing level. Haugh unit, eggshell thickness, and eggshell breaking strength of EEC group were higher than those of control group. Egg yolk was determined for fatty acid profiling. It was found that EEC group had higher ratio of unsaturated- to saturated fatty acid as compared to control group. Lower ratios of n−6 to n−3 fatty acids were found in the egg yolk of EEC group. Plasma triglyceride and total cholesterol contents were lower in the EEC group. However, high density lipoprotein-cholesterol content was higher in the EEC group as compared to that in control group. The number of cecal *Lactobacillus* was increased while the population of *Escherichia coli* and coliform bacteria decreased in the EEC group. Fecal ammonia and hydrogen sulfide contents were lower in the EEC group as compared to those in control group. Taken together, these results suggested that EEC could improve egg production and egg quality. In addition, it could remove odour from laying-hen manure.

Key words

Blood lipid, Cecal microflora, Earthworm cast, Egg production, Egg quality

Introduction

Among air pollution, odour is a hard nut to crack and there are different types of odour. As eco-friendly animal management is strongly required to conserve the natural environment and continuously manage animal husbandry, technology to reduce the odour generated by manure in livestock stables is emerging as an important issue. From the perspective of animal welfare, as well as, health of the livestock and the breeder, improvement of animal husbandry environment through removal of odourants inside and outside livestock stables is required. odourants are a source of environmental pollution that stimulates a sense of smell even at low concentrations. The odour generated in livestock stables, in particular, are a target of public grievance. As environmental purification organisms, earthworms are known as excellent bioconversion agents for efficient treatment of organic waste resources such as food wastes and livestock feces (Kim *et al.,* 2000; Matta *et al.*, 2008).

Earthworm casts contain undecomposed organic materials excreted after feed is ingested by earthworms then decomposed and absorbed in their digestive tracts. Since antibiotic bacteria like *Bacillus* sp. in earthworm casts suppress and dissipate the activities of mold that are harmful to the soil, the casts are used as eco-friendly fertilizers for golf

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course grass lawns (Chaoui *et al.,* 2003). They contain high amount of nitrogen, phosphoric acid, potassium etc., and provide high deodourizing effects due to their excellent aeration and positive ion exchange capacities (Sinha *et al.,* 2009). They are evaluated as excellent biological resources for removing odour in livestock stables and produce eco-friendly and safe livestock products if microbial fermentation conditions are provided (Yoo and Lee, 2007).

Accumulation of manure from livestock feeding and management has given rise to environmental pollution in stock farms due to odour and leachates. Odour generated in sewage processing facilities, excrement disposal facilities and livestock waste treatment facilities contain various types of odourants. In particular, the concentration of odourants generated from muck increase in summer and the increase in ammonia and hydrogen sulfide generation coupled with the reaction products result in serious odour problem(Bae and Park, 2011).

Removal of NH_3 (Lim and Lee, 2007), H_2S and sulfurous acid gas using earthworm cast biofilters has been reported. Earthworm and *Beauveria bassiana* mixed feed (Lee and Park, 2006), earthworm and photosynthetic bacteria mixed feed (Ahn *et al.,* 2007), fermented- earthworm cast (FEC) (Patent, 2007) and manufacture of poultry feed additives (Patent, 2009) for livestock have also been reported. Earthworms are well-known to be able to eliminate pathogens such as *E. coli, Salmonella* and other harmful organisms in biosolids as safe materials in plant and animal diets (Eastman *et al.,* 2001; Sinha *et al.,* 2009).

However, biological treatments involving addition of fermented-earthworm casts to feed for laying hens have rarely been reported as a method to reduce odour in egg production and livestock facilities.

In light of the above, the present study aimed to assess egg production and egg quality, as well as, removal of harmful odours generated from feces by feeding dietary FEC to laying hens.

Materials and Methods

Experimental animals and design : All the scientific procedures involving animals followed the scientific and ethical procedures presented by Swanson (2008). The experiment protocol using animals was approved by the Institutional Animal Care and Use Committee of Kangwon National University, South Korea., (Approved No: KW-150113-1). Two hundred laying hens (Hyline Brown) were bought from a commercial laying hen farm at 33 weeks of age. They were randomly assigned to 200 individual cages with separate feeders and nipple waterers. All the birds were kept in an environmentally controlled house with light traps without outside light. The temperature was maintained at 23°C throughout the experimental period of 42 weeks. Diets and water were provided to birds *ad libitum*. Each individual hen was an experimental unit. Therefore, there were 100 replications per treatment. Birds were fed with commercial diet formulated to meet nutrient requirements recommended by the Korean feeding standard for poultry. The EEC for this experiment was provided by Huinbio (Co., Ltd. Kimpo, South Korea). Its safety in destroing pathogens such as *E. Coli, Salmonella, Campylobacter* and *Staphylococcus* after 1500! heat treatment has been reported (Patent, 2007). The control group was not treated with EEC The EEC group was treated with top dressing containing 3.5% EEC. Selection of 3.5% EEC was based on the preliminary results that egg production did not increase further when more than 3.5% of EEC was added. Daily egg number, egg weight, and egg quality parameters were individually measured and recorded until 42 weeks of age.

Blood lipid profile : The diets for the experimental animals were withdrawn 12 hr before the end of the experiment. Afterward, 20 chickens per treatment group were selected at random and 1 ml blood was collected from the wing vein of each laying hen into heparinized tubes (Becton Dickinson, Franklin Lakes, NJ USA) using hamilton 25 gauge needles. Using a centrifuge with a constant temperature of 4°C, plasma was separated from the blood samples at 3000 rpm for 20 min. Plasma was quickly frozen in liquid nitrogen gas and stored at -20°C until further biochemical analysis. Triglyceride, total cholesterol and high density lipoprotein cholesterol (HDL-C) were analyzed by a commercial enzyme kit (Sigma Co., Ltd. in USA) using an automatic blood analyzer (Autoanalyzer 7150, Hitachi. Tokyo, Japan) (Park, 2011).

Egg production and egg quality : Egg quality was evaluated using eggs produced for five weeks starting from 38 weeks of age, which was 5 weeks after the initiation of the experimental diets, and ending at 42 weeks of age. A total of 20 saleable eggs (no shell defects, cracks, or double yolks) were randomly collected at 12:00 pm from each treatment on weekly basis. Eggshell breaking strength was evaluated by using an eggshell force gauge (Robotmation. Co., Ltd., Japan). Eggshell thickness was measured on the large end, equatorial region and small end, respectively, using a dial pipe gauge (Ozaki MFG. Co., Ltd., Japan). Egg yolk color and Haugh units (HU) were evaluated using an egg multitester (Touhoku Rhythm. Co., Ltd., Japan).

Fatty acids of egg yolk : Analysis of lipid extraction and fatty acid in egg yolk was performed by the method of Park *et al.* (2014). 5 g of egg yolk was added to 200 ml of pre-mixed organic solvent (chloroform: methanol=2:1) with 6 ml of 0.88% KCl. The mixture was stirred and homogenized for 3

min in a homogenizer (Ultra turrax T25, IKL-Labortechnik, Germany). After adding 50 ml of normal saline to the filtered homogeneous material, the lipid layer was separated from the mixed solution by centrifuging for 20 min at 3000 rpm with an automatic refrigerated centrifuge (RC-3, Sorvall Co., USA), maintaining a constant temperature of 4°C. The centrifuged lipid layer was concentrated at 45°C by injecting nitrogen gas with a rotary vacuum evaporator (Rotary evaporator N-100, Eyela., Japan). 10 mg of concentrated lipid fraction was placed in a reaction vessel for saponification. 0.5N of newly made methanolic NaOH 1 ml was added to the reaction vessel. After heating for 15 min, the reaction vessel was cooled again. After cooling, 14% BF_3-methanol 2 ml was added to the reaction vessel and the reaction vessel was heated until the mixed solution was methylated. After cooling the reaction vessel to room temperature, 1 ml heptane and 2 ml NaCl saturated solution were added to the reaction vessel then mixed for 1 min. Afterward, the vessel was left for 30 min at room temperature. 1 µl of the supernatant was taken from the reaction vessel. After injecting the supernatant into a gas chromatographic system (model GC-15A, Shimadzu Corp., Kyoto, Japan) with a flame ionization detector, the fatty acid content was analyzed. The product by Supelco in USA (37 component FAME Mix, Sigma Aldrich Co., St. Louis, MO) was used as a standard solution and nonadecanoic acid (19:0) was used as the internal standard. SP™-2560 capillary GC column (L×I.D. 100 m×0.25 mm, df 0.20 µm omegawax 320 capillary column. USA) was used. The starting temperature was 150°C for 8 min and the final temperature was fixed at 190°C. The temperature increment was 2°C per 2 min. Helium was used as a carrier gas, with a flow of 40 ml per min. The split ratio was 100:1, and the temperatures of the injector and detector were adjusted to 250°C and 265°C, respectively.

Cecal microflora : According to experimental animal euthanasia recommendations (NRC, 2011), chickens can be killed without stress by cervical dislocation. To minimize chickens' stress, the selected chickens were transferred to a separate slaughter house 2 hr prior to slaughter. After cervical dislocation, the carotid arteries were severed immediately and the chickens were exsanguinated for 90 sec. After soaking in hot water (58 - 60°C) for about 4 min, they were passed through a dehairer for 2 min for depilation. In order to examine the intestinal microorganisms immediately after slaughtering, cecum was excised using an anaerobic method and kept on ice before storage under anaerobic conditions in sealed anaerobic jars (Oxoid Basmgstoke, UL) containing AnaeroGen sachets (Oxoid, Hampshire, UK). The contents of the cecum were initially diluted 10 times (1:9, wt./vol.) with sterilized anaerobic physiological saline (phosphorus buffered saline; PBS 0.1 M, pH 7.0) and then, 100 µl of the sample diluted in 10^{-2}-10^{-7} was extracted to cultivate microorganisms. All these procedures were performed in anaerobic conditions in an anaerobic chamber (5% hydrogen, 5% CO, balanced nitrogen). Each diluted sample was cultured in a sterilized plate with selective medium, i.e., *Lactobacillus* SPP. (MRS agar, Oxoid, Basmgstoke, UK), *Salmonella* (SS agar Difco, CM0099), *E. coli* (McConkey purple agar), Coliform bacteria (Violet red bile agar, Difco) and Total aerobic bacteria (Nutrient agar, Difco). Aerobic bacteria were aerobically cultured in incubators at 37°C for 24 hr while anaerobic bacteria were cultured in stationary condition by using incubators at 37°C for 48 hr. The number of colonies in each plate medium was counted with a microorganism counter. Number of microorganism colonies, which were the colony-forming units in the cecum, were represented in common logarithm (Feng *et al.*, 2015).

Determination of fecal NH_3 and H_2S : The densities of NH_3 and H_2S, generated when 500 g of fresh feces from laying hens were sealed in a vinyl bag (20×30 cm) and left for 5 days at 33°C, was measured using a gas indicator (AP-20, Axis Sensitive Co. Ltd, Japan). In accordance with the manufacturer's user manual, the value displayed after inhalation of gas was recorded and compared to that of control group.

Statistical analysis : Analysis of variance for all the obtained data was performed using GLM procedure in SAS software (SAS, 2004). After t-test, statistically significant differences between the processing averages were tested at a reliability level of 95 % ($p<0.05$).

Results and Discussion

Egg production and egg weight in EEC group were significantly ($p < 0.05$) increased as compared to those in control group (Table 1). However, EEC had no effect on weight of egg yolk. Therefore, top dressing with 3.5% EEC in laying-hen diets could improve egg production and egg quality. EEC might have served as a substrate and accelerated the growth of beneficial intestinal bacteria such as *Lactobacillus,* subsequently elevating antibacterial activity and immune capabilities, while simultaneously improving egg production (Park, 2008). Currently, EEC was applied to laying hens.

Egg yolk color and egg composition (HU, eggshell thickness, and eggshell breaking strength) were measured as indicators of egg quality. It was found that HU, eggshell thickness, and eggshell breaking strength were significantly ($p < 0.05$) increased in the EEC group as compared to that in control group (Table 2). However, EEC had no effect on egg yolk color as compared to control group (Table 2). It has been reported that HU, eggshell thickness, eggshell breaking strength and yolk color are important factors for determining

egg quality related to consumer preference (Park *et al*., 2012). HU is the standard for determining the interior egg quality. The rate of quality loss measured by HU is not linear (Keener *et al*., 2006). The improvement of egg quality in the EEC group might be due to increased nutrient absorption and improved calcium absorption. Calcium is one of the main component in the eggshell. Egg yolk color can indicate the lightness and shade of egg yolk color with grades ranging from 1 to 14 (Park *et al*., 2014).

There were significant differences in triglyceride and total cholesterol contents between the two groups, with significantly ($p < 0.05$) lower levels in hens fed with EEC as compared to that in control (Table 3). Laying hens fed with EEC had significantly ($p < 0.05$) higher HDL-C as compared to that in control group.

EEC diet supplementation significantly ($p < 0.05$) increased oleic acid (18:1n-9) and linolenic acid (18:3n-3) contents in the egg yolk compared to the control (Table 4). However, EEC significantly ($p < 0.05$) reduced linoleic acid (18:2n-6) content in the egg yolk (Table 4). The unsaturated fatty acid to saturated fatty acid ratio (UFA/SFA) in the eggs was significantly ($p < 0.05$) higher in the EEC group as compared to that in control group. Concomitantly, n-6/n-3 ratio in the eggs of the EEC group was significantly ($p < 0.05$) lower than that in the eggs of control group. It has been recommended that most Americans should increase their consumption of n-3 fatty acids but decrease the ratio of n-6/n-3 fatty acids {add reference}. Typical n-6/n-3 ratio in eggs fed with common corn-soybean meal diets, diets containing menhaden oil, and diets containing flax are 16:1, 4:1, and 2:1, respectively. The ideal ratio of n-6/n-3 in food for human health is 4:1 or lower (Park *et al*., 2012). The result showed that eggs from EEC-fed hens had a closer to ideal n-6/n-3 ratio than typical eggs, indicating that EEC might be able to contribute to the production of high quality eggs. High ratio of unsaturated fatty acid and low ratio of saturated fatty acid in the EEC group could be related to fatty acid metabolized after organic acid produced in the lower digestive tract enters the liver through portal veil (Park *et al*., 2012).

The number of *Lactobacillus* in hens fed with EEC was 10.5 fold higher than that of hens fed with control diet. However, significantly ($p < 0.05$) lower number of *E. coli* and coliform bacteria were found in the EEC group as compared to that of control group. No difference in the number of aerobic bacteria or *Salmonella* was found between two

Table 1 : Effect of feeding fermented-earthworm casts (EEC) on laying performance of laying hens

	Control	FEC 3.5%
Egg production (%)	92.43±2.56	95.43±3.76*
Egg weight (g)	64.74±2.05	65.79±1.49*
Egg yolk (g)	14.13±0.87	14.77±0.73

*p<0.05 (Means±SD, n=10)

Table 2: Effect of feeding fermented-earthworm casts (EEC) on egg quality

	Control	FEC 3.5%
Haugh unit (HU)	85.06±1.52	88.77±1.69*
Eggshell thickness (mm)	0.33±0.03	0.36±0.02*
Eggshell breaking strength ($kg\ cm^{-2}$)	3.55±0.27	4.23±0.21*
Egg yolk color	9.09±0.12	9.12±0.16

*p<0.05 (Means±SD, n=10)

Table 3: Effect of feeding fermented-earthworm casts (EEC) on plasma lipid profiles of laying hens

($mg\ dL^{-1}$)

	Control	FEC 3.5%
Triglyceride	131.8±8.23*	109.7±10.21
Total cholesterol	80.11±8.09*	76.81±7.16
HDL-cholesterol	10.96±2.83	15.50±3.17*

*p<0.05 (Means±SD, n=10)

Table 4 : Effect of feeding fermented-earthworm casts (EEC) on fatty acid composition of egg yolk

(% of total fatty acid)

Fatty acid name		Control	FEC 3.5%
Formula	Common name		
C8:0	Octanoic acid	-[1)]	-
C10:0	Decanoic acid	-	-
C12:0	Lauric acid	0.22±0.02*	0.001±0.0001
C14:0	Myristic acid	0.76±0.07	1.07±0.06*
C16:0	Palmitic acid	26.41±0.25	26.62±0.17
C16:1n-9	Palmitoleic acid	5.03±0.06	5.67±0.07*
C18:0	Stearic acid	6.65±0.02*	6.27±0.06
C18:1n-9	Oleic acid	43.75±0.80	45.76±0.72*
C18:2n-6	Lnoleic acid	15.21±0.43*	13.86±0.21
C20:0	Arachdic acid	-	-
C18:3n-3	Linolenic acid	0.26±0.05	0.74±0.02*
C22:0	Behenic acid	1.70±0.07*	0.001±0.0001
C22:1	Erucaic aicd	-	-
C24:0	Lignoceric acid	-	-
Total		100	100
SFA		35.74±7.25	35.96±8.07
UFA		64.26±5.36	66.03±5.07*
n-6/n-3		58.50±3.20*	18.73±1.06
UFA/SFA		1.80±0.05	1.94±0.07*

[1)]: Not detected. SFA : Saturated fatty acids. UFA : Unsaturated fatty acids. *p<0.05 (Means±SD, n=10)

Table 5 : Effect of feeding fermented-earthworm casts (EEC) on cecal microbial population of laying hens

(log cfu g^{-1} content)

	Control	FEC 3.5%
Lactobacillus	7.77±0.74	8.17±0.63*
Escherichia coli	6.06±0.22*	4.77±0.19
Coliform bacteria	5.68±0.11*	3.92±0.06
Total aerobic bacteria	6.72±0.17	6.21±0.21
Salmonella	3.89±0.18	3.61±0.16

*p<0.05 (Means±SD, n=10)

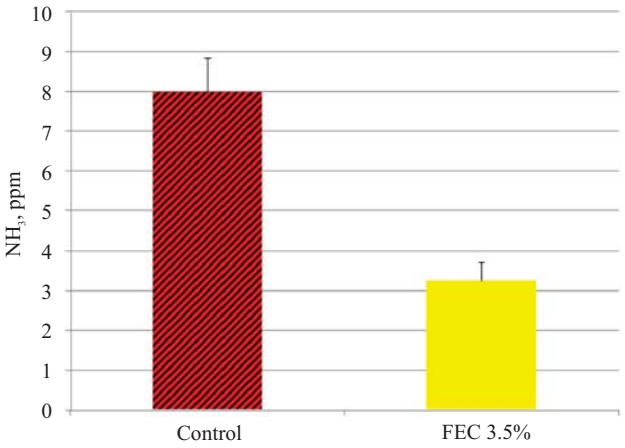

Fig. 1 : Changes in fecal NH3 from laying hens fed fermented-earthworm casts (EEC) during weeks 33 to 42. Bars represent means±SD (*p<0.05, n=10)

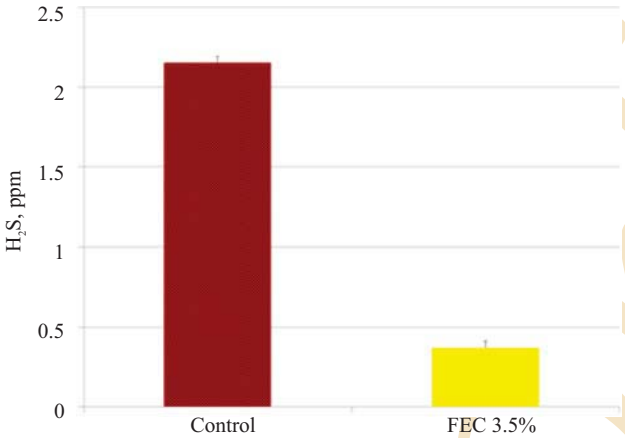

Fig. 2 : Changes in fecal H2S from laying hens fed fermented-earthworm casts (EEC) during weeks 33 to 42. Bars represent means±SD (*p<0.05, n=10)

groups. EEC might have acted as a substrate for the growth of beneficial bacteria such as lactic acid bacteria that possess powerful *in vivo* antibacterial activity in the cecum (Gong *et al.*, 2002; Ahn *et al.*, 2007; Park, 2008; Park *et al.*, 2012). *Lactobacillus* against *E. coli* secretes bacteriocin containing active substances and organic acids such as lactic acid and acetic acid. This mechanism can suppress intestinal colony forming activities by pathogenic bacteria (Rolfe, 2000; Zhang *et al.,* 2003). Intestinal bacteria play a role in synthesizing fermentation products to supply energy for intestinal epithelial cells, stimulating immune system in the digestive tract, synthesizing vitamin K and conferring resistance against the colony forming activity of extrinsic pathogenic bacteria (Tako *et al.,* 2008). *Lactobacillus* is known as a beneficial bacteria to animal health but other bacteria including *E.coli* and *Clostridium perfringens* can be harmful (Devaraj *et al.,* 2002). Since intestinal beneficial microflora such as *Lactobacillus* competes with potential pathogens for nutrient and digestive tract sites, it obstructs the formation of intestinal pathogenic bacteria, secretes bacteriocin, which is a powerful antibiotic substance against *E. coli.* and produces substrates for organic acid and other bacteria. This is likely related to reduction in the number of *E. coli* and *Salmonella* in the cecum of FEC group.

The level of NH_3 and H_2S significantly ($p < 0.05$) decreased in the EEC group as compared to that in control group (Fig. 1 and 2). This study confirmed that EEC could reduce NH_3 and H_2S, the cause of odour in poultry houses. Nitrogen is excreted as uric acid in the urine of poultry or in the form of urea, ammonia and organic nitrogen in animal feces. Conversion of uric acid to ammonia requires enzyme urease that is excreted in animal feces. The breakdown of complex organic nitrogen in the feces occurs slowly. Feces of poultry with further microbial action releases ammonia during manure decomposition. Ammonia can have negative impact on the animal health and production, as well as, human health (Susan, 2009). The odour reduction effect of EEC might be due to increased *Lactobacillus* and reduced harmful bacteria in the cecum. *Lactobacillus* is suspected to be able to produce organic acids such as lactic acid and acetic acid against cecum fermentation. These organic acids can subsequently reduce odour by lowering the pH in the cecum and suppressing the growth of pathogenic bacteria (Zhang *et al.*, 2003). These study results showed that dietary fermented earthworm cast could improve egg production and egg quality. In addition, it could remove odour from laying-hen manure.

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